

# Measurement of dialkyl phosphate metabolites of organophosphorus pesticides in human urine using lyophilization with gas chromatography-tandem mass spectrometry and isotope dilution quantification

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Urinary dialkylphosphate (DAP) metabolites have been used to estimate human exposure to organophosphorus pesticides. We developed a method for quantifying the six DAP urinary metabolites of at least 28 organophosphorus pesticides using lyophilization and chemical derivatization followed by analysis using isotope-dilution gas chromatography–tandem mass spectrometry (GC–MS/MS). Urine samples were spiked with stable isotope analogues of the DAPs and the water was removed from the samples using a lyophilizer. The dried residue was dissolved in acetonitrile and diethyl ether, and the DAPs were chemically derivatized to their respective chloropropyl phosphate esters. The chloropropyl phosphate esters were concentrated, and analyzed using GC–MS/MS. The limits of detection of the method were in the low  $\mu\text{g/l}$  (parts per billion) to mid  $\text{pg/ml}$  range (parts per trillion) with coefficients of variation of 7–14%. The use of stable isotope analogues as internal standards for each of these metabolites allows for sample-specific adjustment for recovery and thus permits a high degree of accuracy and precision. Use of this method with approximately 1100 urine samples collected from pregnant women and children indicate that the low limits of detection allow this method to be used in general population studies.

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## Introduction

Pesticides are used extensively in both agricultural and residential applications worldwide. These current-use pesticides include organophosphorus (OP), carbamates, triazines, chloroacetanilides, and synthetic pyrethroids. Among these, OP pesticides are the most widely used in agriculture and pest-control applications that contribute about 8% to the total amount of pesticides used in the United States (EPA, 1999). Because of the widespread use of OP pesticides, exposure of the general population to these chemicals is almost impossible to avoid. To properly evaluate risks associated with these exposures and to document exposure trends over time, viable techniques for evaluating exposure are required. One technique that has been widely accepted and used in the scientific community is the measurement of

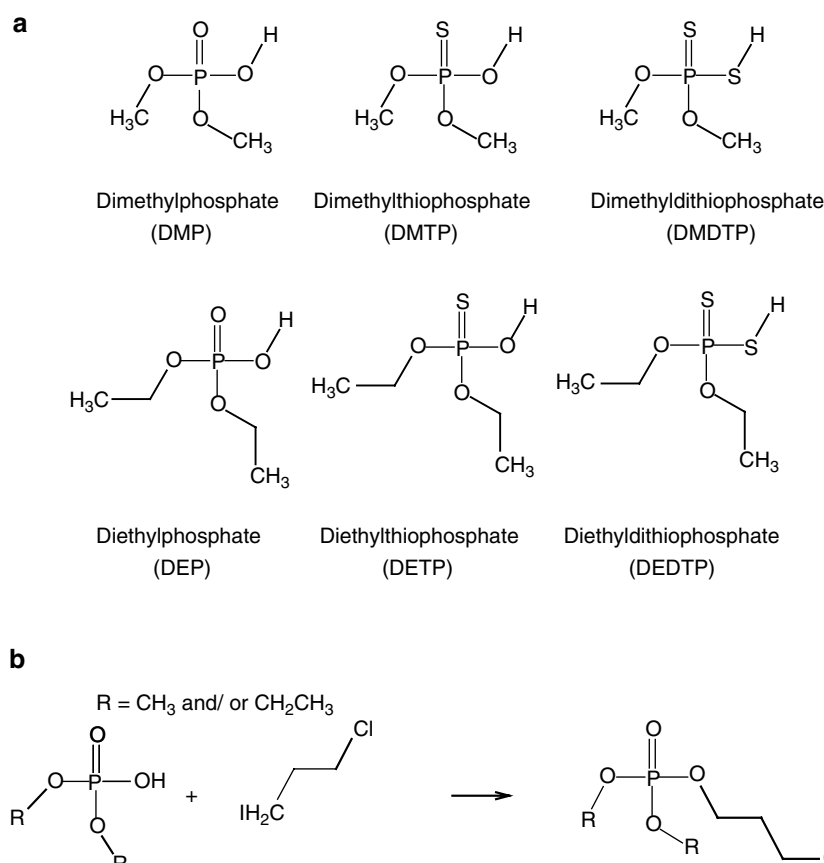
biomarkers of exposure (Needham et al., 1995; Anwar, 1997; Azaroff, 1999; Wessels et al., 2003). However, to produce meaningful data, we must have analytical methods that are accurate, precise, and are adequately sensitive to detect biomarker concentrations resulting from incidental human exposures.

Since the 1970s, urinary dialkylphosphate (DAP) metabolites of OP pesticides have been quantified in human urine as a dosimeter for exposure and bodily adsorption of OP pesticides (Bradway and Shafik, 1977; Reid and Watts, 1981; Bardarov and Mitewa, 1989; Fenske and Leffingwell, 1989; Weisskopf and Seiber, 1989; Drevenkar et al., 1991; Aprea et al., 1994, 1996a, b, 1999, 2000; Davies and Peterson, 1997; Loewenherz et al., 1997; Moate et al., 1999; Simcox et al., 1999. Fenske et al., 2000; Hardt and Angerer, 2000; Heudorf and Angerer, 2001; Lu et al., 2001; Oglobline et al., 2001a, b; Bravo et al., 2002; CDC, 2002; Cocker et al., 2002; Garfitt et al., 2002a, b). The six common DAP metabolites measured are dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP) (Figure 1a). Table 1 shows a list of organophosphorus pesticides approved by the US.

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**Figure 1.** Chemical structures for six dialkylphosphate urinary metabolites (a) and typical derivatization reaction for dialkyl phosphates (b).

Environmental Protection Agency (US EPA) for use and their corresponding phosphorous-containing urinary metabolites. DAP metabolites do not retain the specific chemical structure of the parent pesticides; therefore, individual pesticides from these metabolites are impossible to identify. However, because these metabolites are common to the majority of OP pesticides (Table 1) and laboratory methods are not available for pesticide-specific metabolites for many of these pesticides, the nonspecific DAPs provide valuable information about cumulative exposure to the OP class.

One of the most challenging parts of analyzing DAP metabolites in urine is their isolation from the matrix. Typical isolation or extraction techniques reported in the literature include liquid-liquid extraction using polar solvents (Hardt and Angerer, 2000), solid-phase extraction (SPE) (Lores and Bradway, 1976; Weisskopf and Seiber, 1989; Driskell et al., 1999), azeotropic distillation using acetonitrile:water azeotropes (7:1) (Reid and Watts, 1981; Fenske and Leffingwell, 1989; Moate et al., 1999; Bravo et al., 2002), and lyophilization (Davies and Peterson, 1997; Oglobline et al., 2001 a, b). Because the DAP metabolites are inherently polar with low  $pK_a$ 's, the liquid-liquid

extraction of these metabolites into an organic phase is difficult, resulting in poor recoveries. Other sample preparation techniques, such as SPE and azeotropic distillation, have been evaluated by our laboratory and by other researchers. We found that SPE produced unreliable recoveries that varied greatly depending the concentration of the urine sample and that azeotropic distillation produced more consistent recoveries (Driskell et al., 1999; Bravo et al., 2002). However, the recoveries in azeotropic distillation were typically low, probably because of incomplete dissolution of the residue or binding of DAPs to glass, the residues were extremely dirty, and the residues still contained residual water (Bravo et al., 2002). As a more convenient and robust technique, we considered lyophilization of the urine sample.

In this paper, we describe a simple, selective, sensitive, and repeatable method for measuring six DAP metabolites of OP pesticides in human urine. Urine samples were lyophilized; the residue was dissolved in acetonitrile/ethyl ether and derivatized with 1-chloro-3-iodopropane (Figure 1b). Samples were then analyzed using gas chromatography-tandem mass spectrometry (GC-MS/MS) and quantified using isotope-dilution calibration.

**Table 1.** A total of 28 EPA-registered organophosphorus pesticides and their potential dialkyl phosphate metabolites

Pesticide	DMP	DMTP	DMDTP	DEP	DETP	DEDTP
Azinphos methyl	✓	✓	✓			
Chlorethoxyphos				✓	✓	
Chlorpyrifos				✓	✓	
Chlorpyrifos methyl	✓	✓				
Coumaphos				✓	✓	
Dichlorvos (DDVP)	✓					
Diazinon				✓	✓	
Dicrotophos	✓					
Dimethoate	✓	✓	✓			
Disulfoton				✓	✓	✓
Ethion				✓	✓	✓
Fenitrothion	✓	✓				
Fenthion	✓	✓				
Isazaphos-methyl	✓	✓				
Malathion	✓	✓	✓			
Methidathion	✓	✓	✓			
Methyl parathion	✓	✓				
Naled	✓					
Oxydemeton-methyl	✓	✓				
Parathion				✓	✓	
Phorate				✓	✓	✓
Phosmet	✓	✓	✓			
Pirimiphos-methyl	✓	✓				
Sulfotepp				✓	✓	
Temephos	✓	✓				
Terbufos				✓	✓	✓
Tetrachlorviphos	✓					
Trichlorfon	✓					

DMP = dimethylphosphate; DEP = diethylphosphate; DMTP = dimethylthiophosphate; DMDTP = dimethyldithiophosphate; DETP = diethylthiophosphate; DEDTP = diethyldithiophosphate.

## Experimental section

### Materials

All solvents used were analytical grade with purity greater than 98%. 1-Chloro-3-iodopropane (CIP) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Seven sets of calibration spiking standards in acetonitrile with analyte concentrations ranging from 0.0145 to 1.7 ng/ $\mu$ l were prepared under contract by Battelle Memorial Institute (Bel Air, MD, USA). Native standards of DMP and DEP (98% purity) were purchased from AccuStandard Inc (New Haven, CT, USA) and ChemServices (West Chester, PA, USA), respectively. Although the manufacturer did not determine the purity of DMP, Battelle determined it to be 33% pure using nuclear magnetic resonance analysis. The impurities primarily consisted of the O-monoalkyl substituted phosphate and inorganic phosphate with none of the other five metabolites. DETP (98%) and DEDTP (97%) were both purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Native standards of DMTP (98%) and DMDTP (98%) were purchased from AppliChem (Darmstadt, Germany). No further purifications were performed for the native analytes, but the purities were used to calculate

the final concentration of the native analytes. Isotopically labeled internal standards (ISTD) of DMP (dimethyl- $d_6$ ), DEP (diethyl- $d_{10}$ ), DMTP (dimethyl- $d_6$ ), DMDTP (dimethyl- $d_6$ ), DETP (diethyl- $d_{10}$ ), DEDTP (diethyl- $^{13}C_4$ ) were custom synthesized by Cambridge Isotope Laboratories (Andover, MA, USA) with isotopic purities greater than 99%.

Gases used by the instrumentation had a minimum purity of 99.999% and were purchased from Holox (Atlanta, GA, USA).

Synthetic urine was prepared according to the procedure of Gustafsson and Uzqueda (Gustafsson and Uzqueda, 1978) with bioanalytical grade I water prepared in-house using a Solutions 2000 water purification system (Solution Consultants, Jasper, GA, USA).

### Standard Preparation

**Isotopically Labeled Internal Standard** An isotopically labeled ISTD solution was prepared by weighing approximately 0.5 mg of each isotopically labeled analyte into a 100-ml volumetric flask and dissolving with acetonitrile (HPLC grade, Tedia, Fairfield, OH, USA). The resultant

5-ng/ $\mu$ l solution was divided into 1-ml aliquots and stored at  $-20^{\circ}\text{C}$  until used. This solution was used as an ISTD spiked in all unknown samples, quality control (QC) materials, and calibration standards.

**Calibration Standards** Calibration standards were prepared daily by spiking 2.0 ml of "blank" urine with 10  $\mu$ l of the ISTD solution and with an amount of the appropriate calibration spiking standard solution to produce urinary concentrations ranging from 0.09 to 50  $\mu\text{g/l}$ . The urine samples were prepared for analysis according to the procedure described below.

#### QC Materials

Urine was collected from multiple ( $>30$ ) donors, combined, diluted with water (1:1 v/v) to reduce endogenous levels of the analytes of interest, and mixed overnight at  $20^{\circ}\text{C}$ . After pressure filtering with a 0.2- $\mu\text{m}$  filter capsule, the urine was divided into three pools. The first pool (QC low pool) was spiked with the native standard stock solution to yield an approximate concentration of 8  $\mu\text{g/l}$  for DEP, DMTP, DMDTP, and DETP; 4  $\mu\text{g/l}$  for DMP; and 0.6  $\mu\text{g/l}$  for DEDTP. The second pool (QC high pool) was spiked with the native standard stock solution to yield an approximate concentration of 20  $\mu\text{g/l}$  for DEP, DMTP, DMDTP, and DETP; 10  $\mu\text{g/l}$  for DMP; and 1.3  $\mu\text{g/l}$  for DEDTP. The third pool was not spiked. After being screened for possible endogenous analytes, it was used, along with synthetic urine, as matrix material for calibration standards and blanks. All QC pools were characterized to determine the mean and the 99th and 95th control limits by a consecutive analysis of at least 20 samples from each QC pool. After establishing the control limits of the pools, individual QC samples contained within each analytical run were evaluated for validity using Westgard multirules (Westgard, 2002).

#### Sample Preparation

All urine, reagents, and standards were brought to room temperature. A 2-ml aliquot of urine was pipetted into a 15-ml tube and spiked with 10  $\mu$ l of the ISTD solution to give a urinary ISTD concentration of 25  $\mu\text{g/l}$  for each analyte. After the sample was mixed, it was placed in a commercial lyophilizer system (Labconco, Kansas City, MO, USA). The lyophilizer was operated overnight in the program mode without further manual manipulation. For a run of 50 samples, the samples were initially frozen for 4 h at  $-34^{\circ}\text{C}$  and atmospheric pressure. After the samples were sufficiently frozen, the vacuum was set to 25.5 mT and the samples remained at  $-34^{\circ}\text{C}$  for an additional 4 hours. The samples were then taken to  $-20^{\circ}\text{C}$  for 2 h,  $0^{\circ}\text{C}$  for 1 h, and finally  $20^{\circ}\text{C}$  for 1 h. The following day after completion of the lyophilization process, 2 ml acetonitrile and 2 ml ethyl ether were added to the residue in each sample tube, then vortex mixed for about 1 min. The supernatant was then poured

into a different 15-ml centrifuge tube to separate it from the undissolved residue. The extraction tubes with the undissolved residue were rinsed with another 1 ml acetonitrile, vortex mixed, and combined with the supernatant. The samples were concentrated to approximately 1 ml in about 10 min using a Turbovap LV (Zymark, Hopkinton, MA, USA) at  $30^{\circ}\text{C}$  and 10 psi of nitrogen. The concentrated samples were poured into a 15-ml test tube, which contained a few grains of potassium carbonate. The CIP (50  $\mu$ l) was added to the samples and mixed. The samples were then placed in a dry bath set at  $60^{\circ}\text{C}$  for 3 h. The supernatant was transferred to a clean tube and evaporated to dryness. The samples were reconstituted using 75  $\mu$ l of toluene, transferred to autosampler vials, capped, and stored under refrigeration until analyzed.

#### Instrumental Analysis

**GC Conditions** Samples (1  $\mu$ l) were injected into the gas chromatograph (TraceGC, ThermoQuest, San Jose, CA, USA) by splitless injection using an autosampler (CTC A200s, Carrboro, NC, USA) with an injection purge delay of 60 s. The GC was coupled to a triple quadrupole mass spectrometer (Finnigan TSQ-7000, ThermoFinnigan, San Jose, CA, USA). A 30-m J & W (Folsom, CA, USA) DB-5MS ([5% phenyl]-methyl polysiloxane, 0.25  $\mu\text{m}$  film thickness, 0.25 mm i.d.) capillary column was used for separation of the chloropropyl phosphate esters. A guard column (deactivated fused silica column, Restek, Bellefonte, PA, USA) was used to help extend the useful life span of the analytical column. The temperatures of the injector and transfer line were  $250^{\circ}\text{C}$ . The column temperature was initially  $80^{\circ}\text{C}$  for 2 min and was then heated linearly to  $250^{\circ}\text{C}$  at  $17^{\circ}\text{C/min}$ . The final temperature of  $250^{\circ}\text{C}$  was held for 2 min.

**Mass Spectrometric Conditions** The chloropropyl phosphate esters were analyzed using multiple reaction monitoring (MRM). All of the precursor ions were the pseudomolecular ( $[\text{M} + \text{H}]^{+}$ ) ions produced by chemical ionization in the positive ion mode. Methane was used as a reagent gas with a pressure of 1500 mT and argon as a collision-induced dissociation gas with a pressure of 2 mT. A full autotune of the mass spectrometer was performed before analysis of every set of samples. MS conditions were as follows: source temperature was  $150^{\circ}\text{C}$ , electron energy was 200 eV, and the potential for the continuous dynode electron multiplier varied depending upon multiplier lifetime. Table 2 summarizes the characteristic precursor/product ion combinations and collision offsets used in measuring each analyte and ISTD with a width mass window of 0.4 amu and a scan rate of  $0.03 \text{ s}^{-1}$ . The product ions for  $^{35}\text{Cl}$  precursor ions were selected to maximize specificity, sensitivity, and linear dynamic range. The product ions for  $^{37}\text{Cl}$  precursor ions

**Table 2.** Multiple reaction monitoring analysis of dialkylphosphate metabolites of organophosphorus pesticides.

Metabolite	CO (eV)	<sup>35</sup> Cl Quantification pair		<sup>37</sup> Cl Confirmation pair		Possible fragment (MH-R) <sup>+</sup> for the quantification ion
		Precursor ion	Product ion	Precursor ion	Product ion	
DMP	-12	203	127	205	127	(MH-C <sub>3</sub> H <sub>5</sub> Cl) <sup>+</sup>
DMP (dimethyl-d <sub>6</sub> )	-12	209	133	211	133	(MH-C <sub>3</sub> H <sub>5</sub> Cl) <sup>+</sup>
DEP	-13	231	127	233	127	(MH-C <sub>5</sub> H <sub>9</sub> Cl) <sup>+</sup>
DEP (diethyl-d <sub>10</sub> )	-13	241	133	243	133	(MH-C <sub>5</sub> H <sub>5</sub> D <sub>4</sub> Cl) <sup>+</sup>
DMTP	-13	219	143	221	143	(MH-C <sub>3</sub> H <sub>5</sub> Cl) <sup>+</sup>
DMTP (dimethyl-d <sub>6</sub> )	-13	225	149	227	149	(MH-C <sub>3</sub> H <sub>5</sub> Cl) <sup>+</sup>
DMDTP	-10	235	125	237	125	(MH-C <sub>3</sub> H <sub>7</sub> ClS) <sup>+</sup>
DMDTP (dimethyl-d <sub>6</sub> )	-10	241	131	243	131	(MH-C <sub>3</sub> H <sub>7</sub> ClS) <sup>+</sup>
DETP	-12	247	191	249	193	(MH-C <sub>4</sub> H <sub>8</sub> ) <sup>+</sup>
DETP (diethyl-d <sub>10</sub> )	-12	257	193	259	195	(MH-C <sub>4</sub> H <sub>8</sub> ) <sup>+</sup>
DEDTP	-12	263	153	265	153	(MH-C <sub>3</sub> H <sub>7</sub> ClS) <sup>+</sup>
DEDTP (diethyl- <sup>13</sup> C <sub>4</sub> )	-12	267	157	269	157	(MH- <sup>12</sup> C <sub>3</sub> H <sub>7</sub> ClS) <sup>+</sup>

CO = collision offset; eV = electron volts.

were used only for confirmation purposes and added to the selectivity of the analysis.

**Data Processing and Analysis** Peaks were automatically integrated using the Xcalibur<sup>®</sup> software (version 1.3) provided with the MS. The background signal was subtracted, and all data were smoothed (3-point smooth). The analyst checked and corrected any discrepancies in peak selection yielding an accurate integration. Peak areas and other pertinent data were exported into a Microsoft EXCEL<sup>®</sup> file and loaded into a Microsoft ACCESS<sup>®</sup> database for permanent storage. All statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC, USA).

**Quantification** Calibration plots were constructed for each analytical run, with seven analyte concentrations (urinary concentrations ranging from 0.09 to 1.7 µg/l) plotted against the response factors. Response factors were calculated as the area of the native analyte ion divided by the area of the labeled analyte ion. Calibration standard concentrations encompassed the entire linear range of the analysis. The lowest standard concentrations were at or below the limits of detection (LOD) to ensure linearity and accuracy at the low concentration end. A linear regression analysis of the calibration plot provided a slope and intercept from which unknown sample concentrations could be determined. Except for DMP, the y-intercepts were statistically indistinguishable from zero.

#### Method Validation

**Daily Operating Protocol** A typical sample batch included one reagent blank, 36 unknown samples, one low QC, one

high QC, and seven standards. Before daily instrumental analysis, a known standard was analyzed to confirm acceptable chromatographic resolution and mass spectral sensitivity. We required that the reagent blank be free of analytes and QC samples pass the Westgard multirules before we approved as valid a batch of sample data.

**Limits of Detection** The LOD was calculated for each analyte as 3s<sub>0</sub>, where s<sub>0</sub> is the standard deviation at zero concentration. S<sub>0</sub> was estimated as the y-intercept of a linear regression analysis of a plot of the standard deviation (in units of concentration) versus the concentrations of the four lowest standards (Taylor, 1987). The calculated LODs were verified by the analysis of similar concentrations of analytes spiked into urine.

**Absolute Analyte Recoveries** The recovery of the method was determined at two concentrations, 10 and 50 µg/l, by spiking six “blank” urine samples (2.0 ml) with the appropriate native standard spiking solution and lyophilizing according to the method. Six additional “blank” urine samples (unspiked) were lyophilized concurrently. After the drying step, all the extracts were spiked with a known amount of the ISTD solution to correct for instrument variation. This resulted in a more accurate recovery calculation. The samples that were not spiked before preparation were then spiked with the appropriate native standard spiking solution to serve as control samples representative of 100% recovery. Samples were derivatized, evaporated, and reconstituted with toluene. Then, the samples were analyzed. The recovery was calculated by a comparison of the ratio of the native standard and ISTD areas in the recovery samples to those in the control samples.

**Relative Recoveries** The relative recoveries of the method were determined by spiking "blank" urine samples, at different concentrations and calculating the concentrations using this method. The relative recovery was determined at each of the concentration levels by comparing the measured concentrations with the expected, or spiked, concentrations. A ratio of 1.00 indicated 100% relative recovery.

**Accuracy** The absolute accuracy of this method is difficult to determine because no reference materials were available for evaluation. However, we linked the results of this method to our previous method by analyzing, in parallel, a series of QC materials and several unknown samples representing a wide concentration range using both methods.

**Selectivity** No objective way exists to determine the degree of selectivity of an analytical method. However, as a matter of consensus, chromatographic separation coupled with MS/MS is considered the most selective analytical technique available (Persson and Vessman McDowall, 1998). The use of a chlorinated derivatizing agent allowed us to analyze  $^{35}\text{Cl}$  and  $^{37}\text{Cl}$  fragments for greater selectivity. The use of the isotope-dilution technique, which provides a chromatographic reference for peak selection, adds another degree of selectivity to the method. Apparent interferences in the form of extra chromatographic peaks or peak shoulders were rarely encountered. Unknown interferences typically resulted in an increased background signal.

**Precision** The method precision was determined by calculating the coefficient of variation (CV) of repeat measurements of the QC materials at two concentrations (about 8 and 20  $\mu\text{g/l}$ ). At least 63 repeat measurements over a 2-month period were used in the calculations.

**Human Studies** Urine samples were collected as part of a study conducted by Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) at the University of California at Berkeley (Eskenazi et al., 1999). This study is multifaceted, with one portion focusing on pesticide exposures in Latina women and their children in the Salinas Valley in southern California. Upon collection, samples were frozen within 4 h and were stored at  $-20^{\circ}\text{C}$  before analysis. All protocols were reviewed and approved by a human subjects review committee and complied with all institutional guidelines for the protection of human subjects. Approximately 1100 urine samples from adults and children were analyzed using this method to validate the speed and ruggedness of the analysis. About 5% of the samples tested were QC materials inserted blindly among the study samples. These QC materials were thawed in the field, aliquoted into regular unknown sample vials, then shipped with the samples on dry ice to CDC.

## Results and Discussion

The measurement of the DAP metabolites of OP pesticides in urine is challenging because of their chemical nature. The polar DAPs are extremely difficult to extract from the polar urine matrix. A variety of methods reported in the literature have noted these difficulties and generally have opted to use polar extraction solvents such as ethyl acetate and diethyl ether or the azeotropic distillation of urine. We have evaluated several sample preparation techniques in our laboratory including liquid-liquid extraction, SPE, azeotropic distillation and lyophilization. We previously described the advantages of azeotropic distillation over SPE and liquid-liquid extraction (Bravo et al., 2002). Among these methods, the azeotropic distillation produced the most consistent results. In addition, we discussed some of the disadvantages of azeotropic distillation, such as relatively low recoveries (17–65%) of the analytes, the labor intensiveness of the technique, and the extremely unclear samples produced. For example, a gummy syrup is produced after azeotropic distillation. Moate et al. (1999) speculated that the alkylation of OP metabolites could be hindered by encapsulation of DAP metabolites in this gummy syrup. They found that a mixture of acetonitrile and dimethylformamide could dissolve the gummy residue and yield a better derivatization of DMP and DEP. However, they did not note any affect on the cleanliness of the sample. Davies and Peterson (1997) and Oglobline et al. (2001) showed that lyophilization was a viable technique for removing water from urine samples for DAP analysis. In addition, Whyatt and Barr (2001) noted that lyophilization was a useful sample preparation tool for DAP analysis in meconium samples. We used lyophilization to resolve the problems caused by the gummy residue by removing the water from the sample, but it also provided many other analytical benefits.

Even though the lyophilization process could take approximately 10 h per batch of 50 samples, it can be performed overnight and does not require direct analyst attention or special laboratory skills. Lyophilization of the urine samples yielded the driest possible sample with high and repeatable DAP recoveries. The gummy residue previously reported from azeotropic distillation was not formed. Even though azeotropic distillation and lyophilization both provided suitable sensitivity to measure DAPs in urine samples collected from individuals with background OP exposures, our lyophilization method has several distinct advantages over the azeotropic distillation. By using lyophilization, we achieved much better recoveries (75–100%) than those by azeotropic distillation (17–65%). This probably contributed to the approximate 50% decrease in the method LODs. Because the sensitivity was greater using lyophilization, it allowed us to use about half the volume of urine than was previously required. Consequently, the amount of material from the urine that was injected into the GC column was less, which kept the instrument cleaner. For example, when

lyophilization was used, the mass spectrometer maintained a good operating sensitivity over a run of at least 50 samples. However, with samples prepared using azeotropic distillation, fewer than 25 samples could be run without a dramatic loss in sensitivity. Also, because of the unattended overnight lyophilization, the newer method was less labor-intensive.

In addition to evaluating various sample preparation techniques, we optimized many parameters of the analytic procedure including solvents for redissolving the lyophilized residue, the derivatization conditions, and mass spectral parameters. Previously, we found that acetonitrile was the best solvent for derivatization of the OP metabolites with CIP. We evaluated several solvent mixtures to maximize the dissolution of the lyophilized residue and to facilitate a more rapid evaporation. The overall recoveries for the solvent mixtures were greater than 75% for all the metabolites (Table 3), indicating that any mixture could be used for the dissolution step. However, we used a combination of diethyl ether and acetonitrile, which sped up the evaporation process. In any case, the recoveries using lyophilization coupled with lower boiling solvents were dramatically higher than with acetonitrile codistillation.

Because the alkylation of DAP metabolites is a thermosensitive reaction and the sulfur-containing DAPs degrade at high temperatures (Fenske and Leffingwell, 1989; Moate et al., 1999; Oglobline et al., 2001 a, b), optimization of the reaction temperature and time is critical. Typically, the sulfur-containing molecules (DMTP, DMDTP, DETP, and DEDTP) react at room temperature while DMP and DEP often require high temperatures to react. Several methods use a two-step derivatization process to accommodate both groups of DAPs (Reid and Watts, 1981; Aprea et al., 1996a, b; Moate et al., 1999). Because performing two separate derivatization reactions is cumbersome and tedious, we sought to achieve an efficient single-step reaction that was effective in derivatizing DMP and DEP but did not degrade the other DAPs. Hardt and Angerer (2000) and Oglobline

et al. (2001a, b) both reported lower temperature derivatizations that required only one step; however, they required long reaction times ranging from 4 h to overnight. We evaluated different temperatures and reaction times to find the best overall reaction conditions for all of the metabolites. The derivatization reaction conditions of 60°C and 3 h offered the best compromise between the full reaction of DMP and DEP and the loss of sulfur-containing compounds. Unlike other reported methods, by using isotopically labeled internal standards, we automatically corrected for the small losses of sulfur-containing DAPs so accurate quantification could be performed.

Although most methods in the literature use GC with a flame photometric detector (Reid and Watts, 1981; Aprea et al., 1996a, b; Moate et al., 1999) or GC-MS (Hardt and Angerer, 2000), we took advantage of the specificity that GC-MS/MS offered. GC-MS/MS is highly selective and allows for the use of isotopically labeled internal standards instead of surrogates. Although one recent method also reported the use of GC-MS/MS for measuring DAPs (Oglobline et al., 2001 a, b), this method did not use labeled internal standards, which enhance the selectivity of the method. For an analyte to be positively detected, three conditions are required: (1) the analyte must coelute on the GC column with the labeled standard; (2) it must have a specified precursor ion; and (3) it must have a specified product ion. Additionally, because we incorporated chlorine into the derivatized phosphate esters, an additional precursor ion 2 amu greater than the primary ion is produced in an approximate 30% abundance, representing the naturally occurring abundance of  $^{37}\text{Cl}$ . This ion can be used to confirm positively detected analytes.

Typical ion chromatograms (RIC) of the native (a) and labeled (b) DAPs in an unknown urine sample are shown in Figure 2. The clarity of this chromatogram is typical of an unknown sample, although most samples do not have detectable levels of all DAPs. All of the analytes were chromatographically resolved and eluted at 6–11 min.

A typical calibration plot for DEP is shown in Figure 3. The plot is linear from 0.1 to 50  $\mu\text{g/l}$ . The  $r^2$  value for the linear regression analysis is 0.995 and the error about the slope is less than 2%. This plot is similar to those obtained for the other analytes. The calibration plot specifications for all DAPs are shown in Table 4.

The relative recoveries of the analytes are shown in Table 4. As the data demonstrate, we consistently calculated the DAP concentration within  $\pm 2\%$  of the spiked concentration.

To further validate our new method and to allow data comparison among existing study data, we positively linked the new method to our previous method. Plots of concentrations analyzed in duplicate samples using the two methods at high [a] and low [b] concentrations are shown in Figure 4. For presentation purposes, all DAPs were plotted on the same graph; however, individual plots of each DAP

**Table 3.** Recovery of metabolites in 2 ml of urine using different mixtures of solvents.

Metabolite	Recovery (%) ( $n = 3$ )			
	Acetonitrile <sup>a</sup> 82.0°C <sup>b</sup>	Methanol <sup>a</sup> 64.6°C <sup>b</sup>	Dichloromethane <sup>a</sup> 84.2°C <sup>b</sup>	Ethyl ether <sup>a</sup> 34.6°C <sup>b</sup>
DMP <sup>c</sup>	90 $\pm$ 20	121 $\pm$ 8	92 $\pm$ 11	95 $\pm$ 10
DMTP <sup>d</sup>	99 $\pm$ 7	91 $\pm$ 15	96 $\pm$ 8	99 $\pm$ 7
DMDTP <sup>d</sup>	98 $\pm$ 4	82 $\pm$ 9	77 $\pm$ 13	82 $\pm$ 10
DEP <sup>d</sup>	85 $\pm$ 8	96 $\pm$ 7	84 $\pm$ 6	82 $\pm$ 11
DETP <sup>d</sup>	89 $\pm$ 5	92 $\pm$ 3	89 $\pm$ 17	87 $\pm$ 9
DEDTP <sup>d</sup>	89 $\pm$ 7	76 $\pm$ 6	78 $\pm$ 5	85 $\pm$ 3

<sup>a</sup>Mixture of 2 ml of acetonitrile with 2 ml of other solvent.

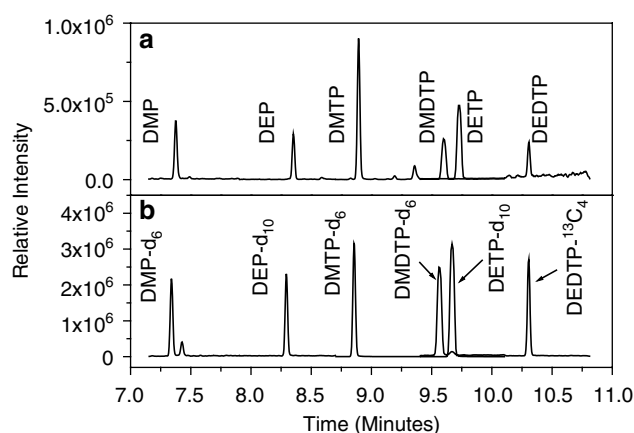
<sup>b</sup>Boiling point.

<sup>c</sup>Recovery at 20  $\mu\text{g/l}$ .

<sup>d</sup>Recovery at 50  $\mu\text{g/l}$ .

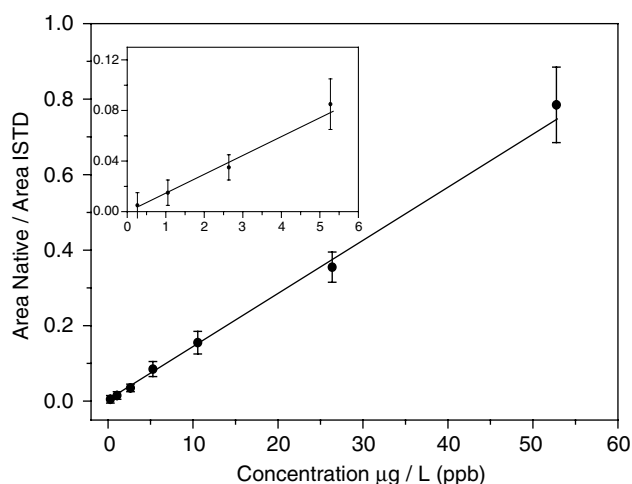
**Table 4.** Summary of the method specifications.

Analyte	$r^2$	% Error of slope <sup>a</sup>	LOD ( $\mu\text{g/l}$ )	Absolute recovery ( $\mu\text{g/l}$ )		Relative recovery <sup>b</sup>	Coefficient of variation (% CV)	
				10	50		Low pool	High pool
DMP	0.992	1.6	0.6	94 $\pm$ 8	95 $\pm$ 10	99.8	10.1	13.6
DEP	0.994	1.6	0.2	99 $\pm$ 4	99 $\pm$ 4	99.6	8.3	7.6
DMTP	0.995	2.1	0.2	100 $\pm$ 11	82 $\pm$ 10	98.6	9.2	9.7
DMDTP	0.990	1.3	0.1	100 $\pm$ 4	82 $\pm$ 11	99.1	8.8	7.5
DETP	0.997	1.2	0.1	82 $\pm$ 6	87 $\pm$ 9	99.8	11.6	7.4
DEDTP	0.999	1.3	0.1	75 $\pm$ 3	85 $\pm$ 3	99.2	13.5	9.0

<sup>a</sup>Error about slope of a linear regression analysis of a calibration plot.<sup>b</sup>Expressed as the slope of a percentage of the spiked concentration.**Figure 2.** Typical chromatogram of the DAP chloropropyl esters and their respective labeled internal standard in an unknown urine sample. Panel a shows the individual MRM traces for each native analyte. Panel b shows the individual MRM traces for each labeled internal standard. To present all peaks in the same scale, the intensity of the DEDTP (panel a) was increased by 10 and the intensity of the DETP-d<sub>10</sub> (panel b) was decreased by 10. The concentrations of the DAP metabolites in the sample are DMP 3  $\mu\text{g/l}$ , DEDTP 0.65  $\mu\text{g/l}$ , the other DAPs range from 6 to 7  $\mu\text{g/l}$ .

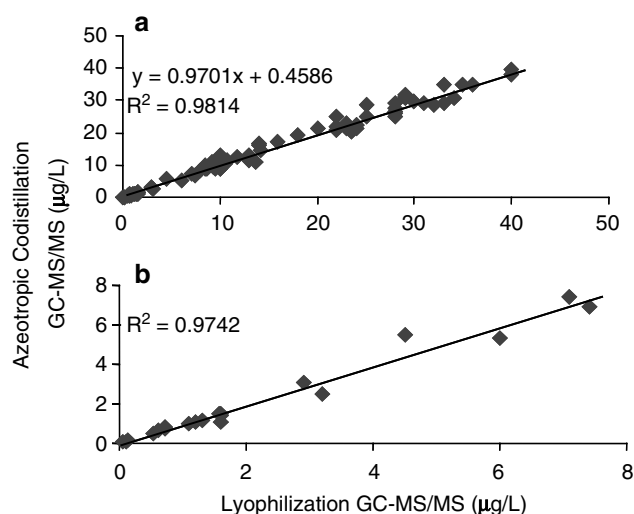
produced similar results. The slope of the best-fit line was 0.970, indicating less than 3% bias between the two methods. The bias was random, not systematic. A Pearson correlation analysis showed excellent agreement between the two methods ( $r=0.99$ ,  $P=0.0001$ ). When only the lower concentrations were considered (inset), the slope and correlation analysis results were still similar, indicating good agreement at low concentrations.

Figure 5 shows the spiked DAP metabolite concentrations plotted against the measured DAP concentrations ( $\mu\text{g/l}$ ) in the blinded QC samples inserted among study samples. The circles represent the data points, and the line represents a linear regression of the data. The correlation coefficient ( $r^2$ ) and the slope of the linear regression line were 0.9827 and 1.000, respectively, indicating excellent agreement between the spiked and the measured values of DEP. The  $r^2$  and slopes of similar plots for the others metabolites are as

**Figure 3.** A typical standard calibration plot for DEP. The plot showed good linearity over the entire calibration range. The correlation coefficient was 0.994 and the standard error was 1.4.

follows: DMP 0.8713, 0.999; DMTP 0.9952, 0.915; DMDTP 0.9893, 1.134; DETP 0.9892, 0.935; and DEDTP 0.9968, 1.209, respectively. These data suggest that the field samples were not compromised during transport or storage and that laboratory error was not significant.

The LODs of this method ranged from 0.1  $\mu\text{g/l}$  (DMDTP, DETP and DEDTP) to 0.6  $\mu\text{g/l}$  (DMP), with CVs ranging from 7% to 14%. Both the LODs and CVs were much lower than those we achieved in routine analysis when using azeotropic distillation (ranging from 0.25  $\mu\text{g/l}$  for DEDTP to 1.6  $\mu\text{g/l}$  for DMTP) even though the volume of urine used for analysis was cut in half (Bravo et al., 2002). In addition, our LODs were lower than those reported in the literature for most methods, which typically range from about 1 to 20  $\mu\text{g/l}$ . Oglobline et al. (2001a, b) recently reported comparable LODs using similar methodology. However, they did not demonstrate linearity or repeatable detection in this low range; their lowest calibration standard was 100–400 greater than their reported LODs. Our CVs are comparable with

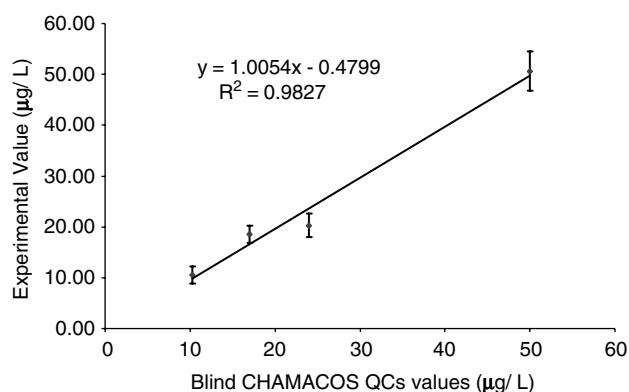


**Figure 4.** Comparison of our two methods for quantifying urinary DAP metabolites of OP pesticides. A Pearson correlation analysis showed good agreement between the two methods ( $r=0.99$ ,  $P=0.0001$ ). Less than 3% of a nonsystematic bias was observed in the results. The inset shows the agreement at the low concentration range.

those reported in the literature for methods with higher LODs. For example, Hardt and Angerer (2000) reported LODs ranging from 1 µg/l for DEP to 5 µg/l for DMP with CVs of less than 17% using a liquid–liquid extraction and GC–MS analysis. Bravo et al. (2002) reported LODs ranging from 1.6 µg/l for DMTP to 0.25 µg/l for DEDTP with CVs of less than 20% using an azeotropic distillation sample preparation and GC–MS/MS analysis. Oglobline et al. (2001a, b) reported LODs of 0.02–0.5 µg/l with CVs ranging from 4 to 14%.

Additionally, high-performance liquid chromatography–tandem mass spectrometry (LC/MS/MS) was explored in an attempt to minimize the sample preparation time by eliminating the 3-h derivatization and the 12-h lyophilization steps. We observed good results with neat standards; however, the sensitivity was inadequate when we spiked them into the urine matrix. We believe the low molecular weights of the DAPs coupled with the non-selective MS/MS losses (i.e., loss of methyl and ethyl groups) increased the chemical noise thus dramatically decreasing our sensitivity. Interestingly, Hernández et al. (2002) reported the determination of DAP metabolites in unextracted urine using LC/MS/MS with LODs of 1 µg/l for DEP, DETP, DEDTP and 2 µg/l for DMTP and CVs lower than 12%. Our observations do not reconcile with this report.

We used our method to measure the six DAP metabolites to evaluate OP exposure in pregnant women and their children in the Salinas valley of California (Eskenazi et al., 2003). One or more of the DAP metabolites was detected in most of the samples tested in this population, confirming that this method has adequate sensitivity to measure DAP



**Figure 5.** A plot of measured values versus spike blind QC samples values in urine (g/l) prepared by CHAMACOS. The circles represent the data points and the line represents a linear regression analysis of the data. The best fit line is  $y = 1.0054x - 0.4799$  and the  $r^2$  value is 0.9827.

metabolites of OP pesticides resulting from environmental exposures. The metabolite most frequently found was DETP, which was detectable in 86% of the samples analyzed, with a geometric mean concentration of 1.6 g/l. The other metabolites were found in the following percentages of the population, with geometric mean concentrations as indicated: DMTP, 81%, 7.0 g/l; DMP, 67%, 2.7 g/l; DEP, 61%, 1.4 g/l; DMTP in 57%, 2.0 g/l; and DEDTP in 23%, 0.1 g/l. At least one DAP metabolite was detected in over 95% of 408 urine samples collected from 6-month old children. These results indicate that our method is suitable for assessing exposures to pregnant women and young children.

## Conclusions

We report a method for quantifying DAP metabolites in urine using lyophilization and chemical derivatization with analysis using isotope-dilution GC–MS/MS. Our method is characterized by its sensitivity, selectivity, and precision. The low analytical LODs of this method allow the determination of internal doses resulting from incidental, or background, exposures. The stability and precision of the measurement system over several months has demonstrated the robustness of the method.

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